ELSEVIER

Biochimica et Biophysica Acta 1310 (1996) 19–24

BBA Biochimica et Biophysica Acta

Effect of K^+-induced depolarization on carbachol-stimulated inositol tetrakisphosphate accumulation in rat cerebrocortical slices

Marvin E. Myles *, John N. Fain

The University of Tennessee, Memphis, Department of Biochemistry, 858 Madison Avenue, Memphis, TN 38163, USA

Received 27 March 1995; accepted 7 July 1995

Abstract

Carbachol-stimulated accumulation of labeled IP_4 or of total Ins 1,3,4,5-P_4 in rat brain cortical slices was maximal in buffer containing 10 mM K^+. Iso-osmotic elevation of extracellular K^+ to 30 mM did not affect total Ins 1,3,4,5-P_4 accumulation but did enhance carbachol stimulated Ins 1,4,5-P_3 accumulation. Iso-osmotically elevated K^+ suppressed carbachol stimulated accumulation of labeled IP_4 while enhancing accumulation of labeled inositol mono-, bis- and trisphosphates. High K^+ alone increased basal accumulation of labeled inositol mono-, bis- and trisphosphates, and total Ins 1,4,5-P_3, while having no significant effect on accumulation of labeled IP_4 or total Ins 1,3,4,5-P_4. Long-term incubation with hyper-osmotically elevated K^+ potentiated carbachol-stimulated Ins 1,3,4,5-P_4 accumulation at 5 min. However, hyper-osmotically elevated K^+ suppressed accumulation of labeled IP_4 due to carbachol. These results indicate that there is no short-term effect of iso-osmotically elevated K^+on carbachol-stimulated total Ins 1,3,4,5-P_4 accumulation. Furthermore, elevating K^+above 10 mM either iso-osmotically or hyper-osmotically suppresses carbachol stimulated accumulation of labeled IP_4. The results suggest that the altered Na^+/K^+ ratio influenced the production of inositol tetrakisphosphates and emphasize the important role of cations such as Na^+, K^+, and Ca^{2+} in the receptor-mediated inositol response. Moreover, the results underscore the unique ability of carbachol (a cholinergic agonist) to stimulate significant accumulation of inositol tetrakisphosphate.

Keywords: Phosphoinositide breakdown; Inositol tetrakisphosphate; Cerebrocortical slice; Muscarinic receptor; Depolarization; Osmolarity

1. Introduction

The regulation of phosphoinositide breakdown in brain is complex. Muscarinic cholinergic agonists directly activate phosphoinositidase C $\beta 1$ through receptor-mediated stimulation of a heterotrimeric (G_Q) protein [1,2]. There is ample evidence that elevation of intracellular Ca^{2+} by any means will also stimulate phosphoinositide breakdown in permeabilized neuroblastoma cells [3], brain slices [4–6] or synaptoneurosomes [7] incubated for 15–60 min with agonists in the presence of lithium after prior labeling with tritiated inositol. Under these conditions the major products seen in the presence of agonists are labeled inositol monophosphates. However, it is now possible at early time periods (2–5 min) to measure total inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) using radioligand binding assays as well as inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P_4). Ins 1,4,5-P_3 is responsible for the release of Ca^{2+} from intracellular compartments [8]. Ins 1,3,4,5-P_4 appears to modulate the entry of Ca^{2+} into cells and acts synergistically with Ins 1,4,5-P_3 in the control of Ca^{2+} homeostasis [8–11].

Ins 1,4,5-P_3 can be metabolized by two different pathways [12,13]. The first consists of a dephosphorylation to inositol 1,4-bisphosphate and inositol 4-monophosphate [12,13]. The second pathway involves the synthesis of Ins 1,3,4,5-P_4 via an Ins 1,4,5-P_3 3-kinase catalyzed phosphorylation of Ins 1,4,5-P_3 [9,14]. Dephosphorylation of Ins 1,3,4,5-P_4 results in, primarily, formation of Ins 1,3,4-P_3 and further degradation to inositol 1-monophosphate [12,13]. The flux through these pathways is influenced by cytosolic concentrations of Ins 1,4,5-P_3, Ca^{2+} and ATP [12]. At early time periods Ins 1,3,4,5-P_4 can accumulate at levels comparable to those of Ins 1,4,5-P_3 [15].

In a previous report [16] we showed that the formation

Abbreviations: Ins 1,4,5-P_3, inositol 1,4,5 trisphosphate; Ins 1,3,4,5-P_4, inositol 1,3,4,5-tetrakisphosphate; IP_4, labeled inositol tetrakisphosphates; IP_3, labeled inositol trisphosphates; $IP_{1\text{-}2}$, labeled inositol mono- and bisphosphates.

* Corresponding author. Fax: +1 (901) 448-7360.

SSDI 0167-4889(95)00130-1

of Ins 1,3,4,5-P_4 in rat brain cortical slices was stimulated only minimally by agents that elevate intracellular Ca^{2+} (i.e., ionomycin, NMDA, ouabain, veratridine). Although depolarization induced by high K^+ (18–40 mM) has been reported to increase phosphoinositide turnover in cerebral preparations [6,17,18], we were not able to detect a significant accumulation of Ins 1,3,4,5-P_4 following exposure of rat brain cortical slices to 30 mM K^+ for periods up to 5 min [16]. Baudry et al. [19] found that elevated K^+ did not inhibit the carbachol response in hippocampal slices, while others [6,18] reported that elevated K^+ (18 mM) actually enhanced carbachol stimulation of labeled inositol tris- and tetrakisphosphate accumulation in cortical slices over a 5-min incubation. Similar effects of high K^+ (20–40 mM) on the accumulation of total Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 were also reported [15]. Our initial attempts to reproduce these findings were unsuccessful. Further efforts to determine the cause of the discrepancy revealed some insights into the possible mechanisms involved in the regulation of Ins 1,3,4,5-P_4 production and/or activity of the Ca^{2+} calmodulin-sensitive 3-kinase that is responsible for phosphorylation of Ins 1,4,5-P_3 to Ins 1,3,4,5-P_4 [9,12,20].

2. Materials and methods

2.1. Preparation of cerebral cortical slices

Male Sprague–Dawley rats (125–175 g) were sacrificed by decapitation and the brains rapidly removed and placed on ice. Three or four rat brains were cleaned of meninges on ice, the cerebral cortices dissected free of white matter and cross-chopped into 350×350 μm slices using a McIlwain tissue chopper (from the Mickle Lab, Engineering Co.). The chopped tissue was incubated in two 50-ml Erlenmeyer flasks each containing 15 ml of Krebs-Henseleit buffer containing 116 mM NaCl, 1.2 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose at pH 7.4 equilibrated with O_2/CO_2 (95:5). The flasks were incubated in a 37°C orbital shaking water bath and shaken at 250 rpm for 15 min. The flasks were removed and the slices washed three times with Krebs-Henseleit buffer then incubated at 37°C and shaken at 250 rpm for 2 h.

2.2. Inositol 1,4,5-trisphosphate and inositol 1,3,4,5 tetrakisphosphate measurements

The slices, after a prior incubation at 37°C for 2 h, were washed three times with Krebs-Henseleit buffer containing 4.7 mM K^+. Increases in medium KCl above 4.7 mM were offset by equivalent decreases in medium NaCl. 50 μl of packed slices were incubated with ligands in a final volume of 150 μl of Krebs-Henseleit buffer for the indicated time periods in triplicate. At end of incubation, 150 μl of ice-cold 1 M TCA was added and the samples kept for 20 min at 4°C. After centrifugation at $2800 \times g$ for 15 min, 200-μl aliquots of the supernatant were removed and washed 4 times with 3 volumes of water-saturated diethylether. A 100-μl aliquot of the washed sample was removed and to this was added 25 μl of 60 mM $NaHCO_3$ and 25 μl of 30 mM EDTA. Aliquots of the neutralized TCA extract were analyzed for inositol 1,4,5 trisphosphate and inositol 1,3,4,5 tetrakisphosphate using binding proteins made from bovine adrenal cortex and pig cerebellum, respectively, as described by Challiss and Nahorski [15]. After addition of the appropriate binding protein, samples were mixed and incubated at 4°C for 30 min with intermittent mixing. Separation of bound and free radioligand was achieved by centrifugation at $13{,}000 \times g$ for 10 min [21]. After centrifugation, the supernatant was aspirated, and the membranes containing bound ligand were solubilized by adding 150 μl of 1% sodium dodecyl sulphate and heating in a boiling water bath for 45 min. After 3 h at room temperature, the contents of the microfuge tubes were transferred to scintillation vials, mixed with scintillant (Ecolume) and radioactivity determined by liquid scintillation counting. Total mass in picomol was determined from the standard binding curves. When necessary, samples were diluted sufficiently to allow mass determination from the most sensitive part of the standard curve. The values are shown as the increments from the zero time values which averaged 0.7 picomol for inositol 1,3,4,5 tetrakisphosphate and 18 picomol for inositol 1,4,5 trisphosphate.

2.3. Measurement of labeled inositol phosphates

Slices were labeled by incubation for 2 h with 2 μCi/ml of [^{3}H]inositol (0.1 mM). Aliquots (50 μl) of gravity packed slices were added to 5-ml plastic tubes containing 100 μl of Krebs-Henseleit buffer and incubated for the indicated times with agonists at 37°C. Inositol phosphates were extracted with percholoric acid and the supernatants were neutralized with potassium hydroxide. The inositol phosphates were separated by chromatography using Dowex AG 1×8 resin [22].

2.4. Materials

[^{3}H]Inositol 1,4,5-trisphosphate, [^{3}H]Inositol 1,3,4,5-tetrakisphosphate and myo-[^{3}H]Inositol were all from Dupont-NEN, Boston, MA; USA. All other chemicals were of analytical grade.

3. Results

Our studies were designed to examine the short-term accumulation of inositol phosphates especially inositol 1,3,4,5-tetrakisphosphate (total Ins 1,3,4,5-P_4 or labeled IP_4), in rat brain cortical slices. All studies were done in

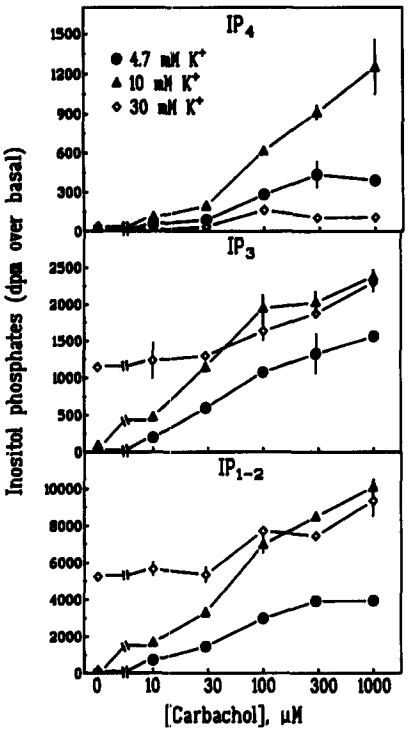

Fig. 1. Dose–response for carbachol-stimulated accumulation of labeled inositol phosphates. Rat brain cortical slices were incubated for 120 min in buffer containing labeled inositol and 1.2 mM Ca^{2+}, rinsed 4 times and incubated for 5 min in buffer containing 1.2 mM Ca^{2+} and either 4.7 mM KCl (circles), 10 mM KCl (triangles) or 30 mM KCl (diamonds) and the indicated concentrations of carbachol. Values represent means ± S.E.M. (triplicate determinations) of the increase over basal values (buffer containing 1.2 mM Ca^{2+} and 4.7 mM K^+) at 5 min. The basal values were: IP_1 and $_2$, 1200 dpm; IP_3, 500 dpm; and IP_4, 185 dpm.

the absence of Li^+ since it does not increase the effects of agonists on the accumulation of tris- and tetrakisphosphates during the first 5 min of incubation, and actually inhibits their accumulation during longer incubations [24,25]. The accumulation over 5 min of labeled IP_4 due to carbachol (Fig. 1) was enhanced in buffer containing 10 mM K^+ as compared to 4.7 mM K^+ as previously shown [15]. For this reason we used 10 mM K^+ in all our studies. In the presence of 10 mM K^+ the addition of carbachol resulted in an increase of labeled IP_4 accumulation (Fig. 1) which was linear between 0.5 and 5 min (data not shown). There was also a rapid accumulation of the isomers of IP_3. The accumulation of labeled IP_4 was at least 50% of the value for inositol trisphosphates. In fact, the increase in labeled inositol tris- and tetrakisphosphates at 5 min (Fig. 1) accounted for one-third of the accumulation of total inositol phosphates in the presence of 1 mM carbachol. We did not separate the isomers of IP_3, but prior studies have indicated that inositol 1,4,5-trisphosphate predominates at early time points, while inositol 1,3,4-trisphosphate increases at later time points since it is derived from breakdown of inositol 1,3,4,5-tetrakisphosphate by a 5-phosphatase enzyme [13]. Elevation of medium K^+ from 4.7 to 10 mM doubled the accumulation of Ins 1,3,4,5-P_4 due to

Table 1
K^+ effects on inositol phosphate accumulation in rat brain cortical slices due to carbachol

Additions	K^+ in mM	Inositol 1,3,4,5-tetrakisphosphate	Inositol 1,4,5-trisphosphate
		Picomoles	Picomoles
None	4.7	< 0.1	1 ± 1
Carbachol, 1 mM	4.7	10 ± 2	37 ± 2
None	10	< 0.1	1 ± 1
Carbachol, 1 mM	10	22 ± 2	39 ± 3
None	30	< 0.1	8 ± 2
Carbachol, 1 mM	30	22 ± 2	61 ± 6

Rat brain cortical slices were incubated for 120 min in buffer containing 1.2 mM Ca^{2+} and 4.7 mM K^+, rinsed 4 times, then incubated for 2 min in buffer containing 1.2 mM Ca^{2+} and either 4.7, 10 or 30 mM K^+. The data are expressed in picomol per tube over the zero time values of 1 picomol for inositol 1,3,4,5-tetrakisphosphate and 20 picomol for inositol 1,4,5-trisphosphate as the mean ± S.E.M. of 4 paired replications.

1 mM carbachol without affecting the increase in Ins 1,4,5-P_3 (Table 1).

In contrast to the reports of others [6,15,17], we found differential effects of high K^+ (30 mM) on carbachol-stimulated accumulation of labeled IP_4 versus accumulation of total Ins 1,3,4,5-P_4. Elevation of extracellular K^+ to 30 mM did not affect Ins 1,3,4,5-P_4 but did enhance carbachol stimulation of Ins 1,4,5-P_3 accumulation (Table 1). Short-term (5 min) exposure of rat brain cortical slices to high K^+ alone resulted in significant accumulation of labeled inositol mono-, bis- and trisphosphates (Fig. 1, Table 2). However, high K^+ did not stimulate accumulation of labeled inositol tetrakisphosphates (Fig. 1, Table 2).

Table 2
Short-term effects of high K^+ on inositol phosphates in rat brain cortical slices. Rat brain cortical slices were incubated for 120 min in buffer containing 1.2 mM Ca^{2+} and 4.7 mM K^+, rinsed 4 times, and incubated for 5 min in buffer containing 10 or 30 mM K^+

	K^+ 30 mM	K^+ 10 mM + Carbachol 1 mM
	Δ from basal	
Labeled inositol mono-, bis- and trisphosphates	+4770 ± 150 [b]	+8460 ± 935 [b]
Labeled inositol tetrakisphosphate(s)	+20 ± 25	+810 ± 105 [b]
Total inositol tetrakisphosphate	−0.5 ± 0.6	+24 ± 3 [b]
Total inositol 1,4,5-trisphosphate	+13 ± 5	+27 ± 2 [b]

The values are the means ± S.E.M. of the paired differences in 5 experiments for total inositol 1,4,5-trisphosphate and inositol 1,3,4,5 tetrakisphosphate accumulation and for 4 experiments when tritiated inositol was present during the initial two-hour incubation. The data are expressed in dpm or picomol per tube due to added agents over the basal values after 5 min incubation in the presence of 10 mM K^+: total inositol 1,3,4,5-tetrakisphosphate, 1 picomol; inositol 1,4,5-trisphosphate, 29 picomol; labeled inositol tetrakisphosphates, 180 dpm; and labeled inositol tris-, bis- and monophosphates, 2000 dpm. Statistically significant differences from basal values are as follows: [a] $P < 0.05$ and [b] $P < 0.01$.

Table 3
Effects of long-term high K^+ on carbachol stimulation of inositol 1,3,4,5-tetrakisphosphate accumulation

Buffer composition	5 min Basal	2 min with 1 mM carbachol	5 minutes with 1 mM carbachol
	Inositol 1,3,4,5-tetrakisphosphate in picomol		
K^+ 10 mM + Na^+ 136 mM	2 ± 1	30 ± 4	34 ± 3
K^+ 30 mM + Na^+ 116 mM	2 ± 1	36 ± 5	33 ± 8
K^+ 30 mM + Na^+ 141 mM	3 ± 1	41 ± 3	183 ± 25

After an initial 15-min incubation, slices were washed and added to flasks in Krebs-Henseleit buffer containing 4.7 mM K^+ for a 60-min incubation. Slices were washed and gased every 20 min for the first 60 min. After the third wash, 50 μl of slices were aliquoted into assay tubes containing the respective buffers without carbachol and incubated for an additional 60 min at 37°C prior to exposure to carbachol for either 2 or 5 min. Data are expressed as picomol per 50-μl slice and represent the mean ± S.E.M. of three experiments.

High K^+ alone at 5 min stimulated total Ins 1,4,5-P_3 accumulation while having no effect on accumulation of Ins 1,3,4,5-P_4 (Table 2). At 5 min carbachol stimulated significant accumulation of labeled and total inositol phosphates (Fig. 1, Table 2).

It was reported [15] that high K^+ (20–40 mM) enhanced the accumulation of Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 at 5 min due to carbachol. In our studies, elevating K + from 10 to 30 mM while reducing Na^+ to maintain iso-osmolarity did not inhibit or potentiate carbachol stimulation at 2 min of Ins 1,3,4,5-P_4 accumulation (Table 1, Table 3). However, with long-term exposure, if we maintained Na^+ at 141 mM while increasing the KCl content of the buffer from 5 to 30 mM we found a marked enhancement of carbachol-stimulated Ins 1,3,4,5-P_4 accumulation, especially at 5 min (Table 3). These results indicate that there is no effect of increasing K^+ from 10 to 30 mM on Ins 1,3,4,5-P_4 accumulation if Na^+ is lowered by the same amount to maintain iso-osmolarity.

The results in Table 3 suggested that it might be the 16% increase in osmolarity due to elevation of K^+ that accounted for the previously reported findings [6,15,17]. To examine this further, we exposed radiolabeled slices to carbachol in buffers that were either isotonic or hypertonic and measured the accumulation of inositol phosphates at 5 min (Table 4). In our studies high K^+ (30 mM) inhibited carbachol stimulated accumulation of labeled inositol tetrakisphosphates regardless of the osmolar status of the buffers used (Fig. 1, Table 4). Similar results were observed at 2 min (data not shown). These effects of high K^+ on labeled (Tables 2 and 4) versus total inositol tetrakisphosphates (Tables 1 and 3) were consistently observed. This suggests that we may be examining different phospholipid pools [6,26]. Alternatively, owing to the complexity of slice preparations, we may be labeling a different cell type from that in which we observed the effects of high K^+ on total Ins 1,3,4,5-P_4.

The data in Fig. 1 demonstrate that in the presence of 30 mM K^+, and at all concentrations of carbachol in the range of 30 to 1000 μM, there were no effects on accumulation of inositol mono, bis- and trisphosphates. However, in the presence of 30 mM K^+ there was no significant accumulation of labeled inositol tetrakisphosphates at any concentration of carbachol (Fig. 1). While one could argue that the effect of high K^+ on labeled IP_4 involves enhanced degradation, we are still left with an apparent lack of an effect on total Ins 1,3,4,5-P_4 (Tables 1 and 3). Thus in our studies a consistent pattern has emerged regarding the stimulation/regulation of inositol tetrakisphosphate ac-

Table 4
Hypertonicity and K^+ effects on basal and carbachol stimulation of accumulation of labeled inositol phosphates

Buffer changes	Basal at 5 min		+ Carbachol at 5 min		
	IP_{1-2}	IP_3	IP_{1-2}	IP_3	IP_4
	% of K^+ 10 mM, Na^+ 136 mM, Cl^- 123 mM				
50 mM sucrose	99 ± 6	151 ± 15 [b]	120 ± 18	131 ± 17	101 ± 10
30 mM K^+ and 116 mM Na^+	257 ± 29 [c]	488 ± 89 [b]	133 ± 12	139 ± 13 [a]	33 ± 13 [a]
30 mM K^+ and 116mM Na^+ plus 50 mM sucrose	399 ± 44 [c]	934 ± 180 [c]	206 ± 19 [c]	212 ± 16 [c]	41 ± 9 [b]
30 mM K^+ and 141 mM Na^+ plus Cl^- 148	273 ± 30 [c]	571 ± 71 [c]	115 ± 10	131 ± 16	37 ± 9 [b]
Na^+ 161 mM and Cl^- 148 mM	54 ± 4 [c]	73 ± 16 [b]	109 ± 15	104 ± 6	88 ± 18

Rat brain cortical slices were incubated for 2 h in the presence of labeled inositol, then the medium was removed prior to addition of the different buffers at room temperature. The medium was changed a total of four times and then the slices were incubated for 5 min at 37°C in either the absence or presence of 1 mM carbachol. The data are the means ± S.E.M. of 5 paired replications (except for inositol tetrakisphosphates which are data from three experiments) and expressed as % of the values in the presence of the standard buffer containing K^+ 10 mM, Na^+ 136 mM and Cl^- 123 mM. The comparison buffers were all hyperosmotic by approximately 50 milliosomol except for the buffer containing 30 mM K^+ where the Na^+ was reduced to 116 mM to maintain isotonicity. The accumulation of inositol mono- and bisphosphates was 2300 dpm in the absence and 7040 dpm in the presence of carbachol at 5 min in the basal buffer, while accumulation of labeled inositol trisphosphates was 260 dpm in the absence and 1365 dpm in the presence of carbachol. Accumulation of labeled inositol tetrakisphosphates was 455 dpm in the presence of carbachol and undetectable in the absence of carbachol. [a] $P < 0.05$; [b] $P < 0.025$; [c] $P < 0.01$.

cumulation versus inositol mono, bis- or trisphosphate accumulation (Fig. 1, Table 4) [16].

4. Discussion

As noted above, a number of studies from Nahorski's laboratory reported that high K^+ (18–40 mM) enhances carbachol-stimulated accumulation of labeled inositol phosphates as well as total Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 in rat cerebral slices [5,6,15,17,18]. We were not able to see any potentiation by 30 mM K^+ of muscarinic receptor-stimulated inositol tetrakisphosphate accumulation (Fig. 1, Tables 1, 3 and 4). We observed a modest potentiation of the muscarinic response on total Ins 1, 4, 5-P_3 (Table 1) and were only able to obtain high values for Ins 1,3,4,5-P_4 following long-term exposure of slices to high K^+ under conditions of high Na^+ (Table 3). The manner (iso-osmotic or hyper-osmotic) in which K^+ was elevated was not made clear in those studies.

Although it is difficult to attribute the effect of high K^+ on accumulation of labeled IP_4 to toxicity, it is a possibility. In astrocytes, increased taurine release was observed when the external [K^+] was raised iso-osmotically by substituting KCl for NaCl, but not when external [K^+] was raised hyper-osmotically by adding KCl directly to the medium [23]. We examined (data not shown) the effect of carbachol, K^+ and osmolar concentration on release of labeled taurine from rat brain cortical slices. As expected iso-osmotically elevated K^+ increased release of labeled taurine while increased osmolarity alone suppressed basal release. Carbachol also stimulated taurine release, which is in agreement with others [27] who reported that carbachol stimulated taurine release in rat sympathetic ganglia. Interestingly, the carbachol (cholinergic)-stimulated release of taurine appeared to have both a nicotinic and muscarinic receptor component [27].

Recent studies in cerebrocortical slices [28] and synaptoneurosomes [7,29,30] examined the role of Na^+ ions in the inositol response elicited by depolarizing stimuli (EAAs, KCl, sodium channel activators) or receptor agonists (carbachol, norepinephrine, etc.). The results indicate that Na^+-influx and/or intracellular sodium may have an important regulatory role in phosphoinositide metabolism [7,28–30]. Furthermore, the data suggest that gradients of K^+, Na^+ and Ca^{2+} [7,28] are all involved in determining the final effect on phosphoinositide turnover. It is proposed that the penultimate effector in this activation process is the Na^+/Ca^{2+} exchanger which regulates internal Ca^{2+} and Ca^{2+} activates phospholipase C [4,7,28–30]. The inability to detect significant accumulation of Ins 1,3,4,5-P_4 with intracellular Ca^{2+}-elevating agents [5,6,16] suggests that carbachol stimulates Ins 1,3,4,5-P_4 accumulation by an independent mechanism(s) or the metabolism of different pools of membrane phosphoinositides [6,16,26,29].

Elevating K^+ either iso-osmotically or hyper-osmotically changes the Na^+/K^+ ratio. Our data suggests that it is the change in the relative concentration of these two ions that is responsible for the effects of high K^+ on labeled IP_4 accumulation. Thus elevating K^+ with or without a change in Na^+ results in inhibition of labeled IP_4 accumulation due to carbachol (Table 4). The ability of hyper-osmotically elevated NaCl (Table 4) to lower basal accumulation of inositol mono-, bis- and trisphosphates would seem to support this argument. Although our Na^+ or K^+ reductions or additions were modest, such changes have been reported to have major effects on release of radiolabeled taurine [23] and high K^+ (30 mM) or EAA stimulated accumulation of inositol phosphates over 20–45 min [29,30].

The marked specificity of the cerebellar binding protein for Ins 1,3,4,5-P_4 as compared to Ins 1,4,5-P_3 (respective IC_{50} values of 3 ± 1 nM and 19 ± 6.7 μM) has been convincingly demonstrated [31,32]. These and other reports [33,34] demonstrated that optimal binding of radiolabeled Ins 1,3,4,5-P_4 occurred using an assay buffer at pH 5.0 containing 25 mM KH_2PO_4. At pH 5, Ins 1,3,4,5-P_4 binding was maximal, whereas radiolabeled Ins 1,4,5-P_3 binding (to the Ins 1,4,5-P_3 binding site) was undetectably low [33,34]. Conversely, radiolabeled Ins 1,4,5-P_3 binding to its binding site was maximal at pH 8.0, while Ins 1,3,4,5-P_4 binding (at this pH) is very low [33,34]. These findings make it highly unlikely that the differential effect of high K^+ on total versus labeled IP_4 can be attributed to cross-reactivity of Ins 1,4,5-P_3 in the Ins 1,3,4,5-P_4 mass assay.

Although residual 3-phosphatase activity could possibly degrade Ins 1,3,4,5-P_4 to Ins 1,4,5-P_3, the acidic assay conditions would prevent Ins 1,4,5-P_3 binding to the Ins 1,4,5-P_3 binding sites present [33]. Likewise, the working conditions used for the binding studies (4°C, addition of 2 mM EDTA in the absence of Mg^{2+}) were designed to reduce/inhibit any residual Mg^{2+}-dependent 5-phosphatase activity which could degrade the label [31,32,35]. In a rat cerebral cortical preparation no significant metabolism occurred during the 30-min assay, with > 98% of radioactivity recovered as Ins 1,3,4,5-P_4 after acid precipitation of the binding protein and HPLC analysis of the neutralized samples [31]. Thus significant interference by Ins 1,4,5-P_3 (or Ins 1,3,4-P_3) in the Ins 1,3,4,5-P_4 mass assay appears unlikely.

Our working hypothesis has been that the formation of Ins 1,3,4,5-P_4 is not simply secondary to elevation of Ca^{2+} and Ins 1,4,5-P_3 but is uniquely regulated. The restricted subcellular localization of the enzyme that converts Ins 1,4,5-P_3 to Ins 1,3,4,5-P_4 would seem to support this argument. The kinase (Ins 1,4,5-P_3 3-kinase) is present at highest concentration in the dendritic spines of neuronal cells [36–38]. The Ins 1,4,5-P_3 kinase is primarily found in the dendritic spines of pyramidal neurons associated with the spine apparatus and plasmalemma with the postsynaptic densities showing the highest amount of the en-

zyme [37]. It was suggested that the functional role of the 3-kinase is related to the postsynaptic processing of signals in dendritic spines of some cerebral neurons [37]. Furthermore, the data suggest that the dendritic spines may be the unique sites in the central nervous system where Ins 1,3,4,5-P_4 may function in the control of local Ca^{2+} circuits and in the development of synaptic plasticity [37].

As alluded to above, muscarinic and α-adrenergic agonists were shown to stimulate phosphoinositide breakdown in radiolabeled synaptoneurosomes [7,29,30]. Synaptoneurosomes consist of synaptosomes (resealed presynaptic vesicles) with attached neurosomes (resealed postsynaptic membranes) [39]. Guinea pig synaptoneurosomal preparations have been used extensively for investigation of the formation of cyclic-AMP [39]. The synaptoneurosome preparation appears to provide a 'cleaner' alternative to brain slices for studying phosphoinositide metabolism in the brain. Thus, fruitful future investigations into the phenomenon presented in this report will most likely require use of a synaptoneurosomal preparation.

Acknowledgements

This work was supported in part by NSF Grant GR DCB 9102372 and by a postdoctoral fellowship to M.E.M. (NIH T32 HLO7641).

References

[1] Cockcroft, S. and Thomas, G.M.H (1992) Biochem. J. 288, 1–14.
[2] Rhee, S.G. and Choi, K.D. (1992) J. Biol. Chem. 267, 12393–12396.
[3] Fisher, S.K., Domask, L.M. and Roland, R.M. (1989) Mol. Pharm. 35, 195–204.
[4] Kendall, D.A. and Nahorski, S.R. (1984) J. Neurochem. 42, 1388–1394.
[5] Baird, J.G. and Nahorski, S.R. (1990) J. Neurochem. 54, 555–561.
[6] Baird, J.G. and Nahorski, S.R. (1990) Biochem. J. 267, 835–838.
[7] Gusovsky, F. and Daly, J.W. (1988) Neuropharmacology 27, 95–105.
[8] Irvine, R.F. (1991) BioEssays 13, 419–427.
[9] Irvine, R.F. and Moor, R.M. (1986) Biochem. J. 240, 917–920.
[10] Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Nature 330, 653–655.
[11] Changya, L., Gallacher, D.V., Irvine, R.F., Potter, B.V.L. and Petersen, O.H. (1989) J. Membrane Biol. 109, 85–93.
[12] Erneux, C. and Takazawa, K. (1991) Trends Pharmacol. Sci. 12, 174–176.
[13] Shears, S.B. (1989) Biochem. J. 260, 313–324.
[14] Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985) Biochem. J. 232, 211–215.
[15] Challiss, R.A. and Nahorski, S.R. (1991) J. Neurochem. 57, 1042–1051.
[16] Myles, M.E. and Fain, J.N. (1994) J. Neurochem. 62, 2333–2339.
[17] Baird, J.G. and Nahorski, S.R. (1986) Biochem. J. 141, 1130–1137.
[18] Baird, J.G. and Nahorski, S.R. (1989) J. Neurochem. 53, 681–685.
[19] Baudry, M. Evans, J. and Lynch, G. (1986) Nature 319, 329–331.
[20] Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F. and Majerus, P.W. (1987) J. Biol. Chem. 262, 2146–2149.
[21] Donie, F. and Reiser, G. (1991) Biochem. Biophys. Res. Commun. 181, 997–1003.
[22] Downes, C.P., Hawkins, P.T. and Irvine, R.F. (1986) Biochem. J. 238, 501–506.
[23] Martin, D.L. and Shain, W. (1993) Neurochem. Res. 18, 437–444.
[24] Nahorski, S.R. and Challiss, R.A.J. (1991) Neurochem. Int. 19, 207–212.
[25] Kennedy, E.D., Challiss, R.A.J., Ragan, C.I. and Nahorski, S.R. (1990) Biochem. J. 267, 781–786.
[26] Challiss, R.A.J., Batty, I.H. and Nahorski, S.R. (1988) Biochem. Biophys. Res. Commun. 157, 684–691.
[27] Waniewski, R.A. and Martin, D.L. (1994) Eur. J. Pharmacol. 260, 113–120.
[28] Benuck, M., Reith, M.E.A. and Lajtha, A. (1989) Neuropharmacology 28, 847–854.
[29] Guiramand, J., Vignes, M., Mayat, E., Lebrum, F., Sassetti, I. and Recasens, M. (1991) J. Neurochem. 57, 1488–1500.
[30] Guiramand, J., Nourigat, A., Sassetti, I. and Recasens, M. (1989) Neurosci. Lett. 98, 222–228.
[31] Challiss, R.A.J. and Nahorski, S.R. (1990) J. Neurochem. 54, 2138–2141.
[32] Donié, F. and Reiser, G. (1989) FEBS Lett. 254, 155–158.
[33] Challiss, R.A.J., Willcocks, A.L., Mulloy, B., Potter, B.V.L. and Nahorski, S.R. (1991) Biochem. J. 274, 861–867.
[34] Enyedi, P., Brown, E. and Williams, G. (1989) Biochem. Biophys. Res. Commun. 159, 200–208.
[35] Connolly, T.M., Bross, T.E. and Majerus, P.W. (1985) J. Biol. Chem. 260, 7868–7874.
[36] Go, M., Uchida, T., Takazawa, K., Endo, T., Erneux, C., Mailleux, P. and Onaya, T. (1993) Neurosci. Lett. 158, 135–138.
[37] Yamada, M., Kakita, A., Mizuguchi, M., Rhee, S.G., Kim, S.U. and Ikuta, F. (1993) Brain Res. 606, 335–340.
[38] Yamada, M., Kakita, A., Mizuguchi, M., Rhee, S.G., Kim, S.U. and Ikuta, F. (1993) Dev. Brain Res. 71, 137–145.
[39] Hollingsworth, E.B., McNeal, E.T., Burton, J.L., Williams, R.J., Daly, J.W. and Creveling, C. (1985) J. Neurosci. 5, 2240–2253.